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The Use of Reversed Phase Cartridges (C_{18}) in Processing Plasma for Analysis of Indomethacin by High Performance Liquid Chromatography

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Summary: The purpose of these studies was to develop a high performance liquid chromatographic (HPLC) assay for plasma levels of indomethacin. A reversed phase (C_{18} Sep-Pak) cartridge was used to process plasma for absorption of indomethacin, the internal standard, and impurities. The recovery of plasma indomethacin and the added internal standard was quantitative from 0.5 ml of plasma. The assay was linear from 50 µg/l to 10 mg/l without concentration of the effluent from the Sep-Pak cartridge. The intra-assay coefficients of variation, for ten injections each to calibrate points at 0.15, 0.30, 0.50 and 1.00 mg/l, were 8.15%, 6.29%, 5.47% and 5.39%, respectively and for duplicates of duplicate calibration points were 7.51%, 6.32%, 4.41%, and 2.05%, respectively. The inter-assay coefficients of variation were 8.49%, 6.48%, 5.10%, and 2.22%, respectively. The sensitivity of the assay can be increased by a 3–5 fold concentration of the effluent from the Sep-Pak and preliminary experiments have indicated that as little as 100 µl of starting plasma can be utilized in the assay.

The assay can be used to determine the concentration of indomethacin in small volumes of plasma. Since C_{18} Sep-Pak cartridges were employed to remove contaminating substances, sensitivity and reproducibility were both high while column longevity and efficiency were excellent.

Die Verwendung von Reversed Phase-Patronen (C_{18}) für die Aufarbeitung von Plasma zur Analyse von Indomethacin durch Hochleistungsflüssigchromatographie

Zusammenfassung: Eine hochleistungsflüssigchromatographische Methode zur Bestimmung von Indomethacin im Plasma wird vorgestellt. Zur Absorption von Indomethacin, internem Standard und Verunreinigungen wurde eine Reversed Phase (C_{18}Sep-Pak)-Patrone verwendet. Die Wiederfindung von Indomethacin und dem hinzugefügten internen Standard war aus 0,5 ml Plasma quantitativ. Die Methode ist ohne Konzentrierung des Effluent aus der Sep-Pak-Patrone von 50 µg/l bis 10 mg/l linear. Die Variationskoeffizienten in der Serie betrugen für 10 Injektionen zu jeder Kalibratorkonzentration von 0,15, 0,30, 0,50 und 1,00 mg/l 8,15, 6,29, 5,47 und 2,05%, für Doppelbestimmungen von jeweils zwei Lösungen jeder Kalibratorkonzentration 7,51, 6,32, 4,41 und 2,05%. Die Variationskoeffizienten von Serie zu Serie betrugen 8,49, 6,48, 5,10 und 2,22%. Die Empfindlichkeit der Methode kann durch 3–5fache Konzentrierung des Effluent der Sep-Pak-Patrone gesteigert werden; Vorversuche ergaben, daß nur 100 µl Plasma als Ausgangsmaterial für die Bestimmung ausreichen.

Die Methode kann zur Bestimmung von Indomethacin in kleinen Plasmavolumina angewandt werden. Da C_{18} Sep-Pak-Patronen zur Entfernung von Begleitsubstanzen angewandt werden, sind Empfindlichkeit und Reproduzierbarkeit hoch, dabei Langlebigkeit und Wirksamkeit hervorragend.

Introduction

Indomethacin, a hydroxyindole derivative which inhibits the enzyme cyclo-oxygenase found in endothelial cells and platelets, is used to treat pre-term infants with patent ductus arteriosus in order to effect closure (1 – 5). Use of the medication is limited by its undesirable effects which include inhibition of platelet aggregation (6), decreased renal water clearance (7 – 10) and displacement of protein bound circulating bilirubin (11, 12). A rapid, reliable method for quantitation of plasma indomethacin would aid in determination of therapeutically effective levels for minimizing dosage of administered drug. The ususal method of processing serum or plasma is to precipitate protein, extract both the indomethacin and internal standard with an organic solvent, then concentrate and assay for indomethacin by a variety of methods (13 – 27).

We established a high performance liquid chromatography (HPLC) assay for indomethacin in human plasma by a method previously reported for rabbit plasma (20). However, limited reproducibilty, suboptimal column efficiency and decreased column life occurred despite frequent column cleaning during our studies. These problems were related to residue from processed plasma samples. Therefore, we investigated the use of reversed phase (C_{18} Sep-Pak) cartridges for processing plasma samples before HPLC analysis of indomethacin. This step allowed excellent reproducibility in the quantitation of indomethacin and markedly increased column efficiency and life with less frequent column cleaning.

Experimental

Materials

All solvents, including those used for sample preparation, were HPLC grade. Chemicals were of the highest purity available and purchased from either Fisher Scientific or American Scientific Products. All reagents were filtered through 0.22 µm Millipore filters and degassed before use. The indomethacin and the internal standard 1-(*p*-fluorobenzoyl)-5-methoxy-2-methylindole acetic acid were obtained from Dr. *Morton Rosenberg* and Dr. *Clement Stone* (Merck, Sharp and Dohme Research Laboratories). The Sep-Pak C_{18} cartridges (three different lot numbers) were purchased from Waters Inc.

A Beckman HPLC model 344 with two 112 pumps and a model 421 controller was used for analysis of samples. The components of the processed plasma samples were separated using a C_{18} Ultrasphere ODS Reversed Phase Column (4.6 mm × 150 mm, Beckman) and a Precolumn (4.6 mm × 25 mm, Beckman) each packed with 5 µm particles. A 20 µl sample injector loop was used with a model 160 absorbance detector (Beckman) at 254 nm. A strip chart recorder (Kipp and Zonen) was used for peak height concentration while an integrator (Spectra-Physics SP4270) with the following settings was used to quantitate areas: Chart Speed (CS) = 1 cm/min; Peak Width (PW) = 18; Peak Threshold (PT) = 25; Integrate Inhibit (II) = 1; Disable Tailing Peak Logic (TP) = 1 (off); Attenuation (AT) for chart = 8 at 0.1 min; Attenuation (AT) for chart = 4 at 4.5 min; Peak Markers (PM) = 1 (On) at 4.5 min; Integrate Inhibit (II) = 0 (start integration) at 4.5 min; End Run (ER) = 1 at 9.5 min.

Method

Plasma for the calibration curve and from study subjects was prepared by collecting blood in Venoject tubes (Terumo Medical Corp.) containing liquid potassium EDTA and potassium sorbate. Tubes were placed on wet ice and immediately subjected to centrifugation at 1200 *g* for 10 minutes at 4 °C. Supernatants were divided into aliquots and stored at −70 °C until used for calibration curves or analyzed for indomethacin levels.

Indomethacin stock solution was prepared by dissolving 1.25 mg in a total of 5.00 ml methanol (final 250 mg/l) and then storing at −70 °C. A daily stock solution was prepared by diluting 50 µl of the freezer stock to 5.00 ml with methanol (final stock concentration 2.5 mg/l) and then storing at 4 °C. Internal standard stock solution was prepared in the same manner as the indomethacin to obtain a concentration of 250 mg/l and stored at −70 °C. For the daily stock, 200 µl was diluted to 5.00 ml with methanol (final concentration of 10 mg/l).

For preparation of the calibration curve, the following procedure was used: 1.0 µg of internal standard (100 µl of 10 mg/l stock) was added to 0.5 ml of plasma and then 0.50 µg, 0.25 µg, 0.15 µg or 0.075 µg (200 µl, 100 µl, 60 µl and 30 µl, respectively of 250 mg/l stock) of indomethacin were added. Each of the four calibration curve samples was prepared in duplicate. The patient samples and calibration curve samples were processed identically and simultaneously.

After addition of the internal standard and indomethacin, the volume of each tube was diluted to exactly 2.5 ml with methanol in H_2O, volume fraction 0.75. The mixture was agitated on a vortex mixer to precipitate the proteins and then spun at 1000 *g* for 30 minutes. The supernatant was collected and acidified with 20 µl of glacial acetic acid and stored at −70 °C.

Further purification of the samples was achieved using C_{18} Sep-Pak cartridges. Prior to sample application, each cartridge was washed sequentially with 2 ml of methanol, 5 ml of water and 2 ml of 15 ml/l acetic acid (pH 3.6). The sample was passed through the Sep-Pak with a plastic syringe followed by addition of 1 ml of 15 ml/l acetic acid (pH 3.6), 2 ml of acetonitrile in 0.1 mol/l acetic acid, volume fraction 0.1 and 0.5 ml of methanol. The internal standard and indomethacin were eluted from the cartridge with 1.5 ml of methanol and collected for analysis. Accurate measurement of solvent volumes for the cartridges was critical, particularly for the methanol elution step, in order to recover indomethacin and the internal standard. Loss of liquid was minimized by laying the cartridge on its side between washes.

When processing smaller amounts of plasma (e. g. 100 µl or 250 µl), the amounts of internal standard, methanol/H_2O, volume fraction 0.75 used for precipitation, and amounts of glacial acetic acid were scaled down accordingly. The sample and Sep-

Pak cartridges were processed as outlined above except that the final 1.5 ml of methanol were concentrated either in an airstream under a fume hood or under vacuum.

In order to completely fill the sample loop, 40 µl of each processed sample were injected into the 20 µl sample loop for analysis. A mobile phase of 45 : 55, 0.1 mol/l acetic acid : acetonitrile with a flow rate of 1 ml/min was used (20). The sensitivity was set at 0.020 absorbance units full scale with detector wavelength at 254 nm.

A calibration curve was derived by plotting the ratio of the area of the indomethacin peak/area of the internal standard peak versus the mg/l of indomethacin added to each standard. The amount of indomethacin in each patient sample was determined in duplicate from the area ratios obtained.

The recoveries of both indomethacin and the standard were calculated by adding the amount present at each calibration point to a total volume of 1.5 ml of methanol. The area counts were then compared directly with the area counts obtained in the 1.5 ml of methanol from Sep-Pak after processing plasma calibration curve standards.

The identification of indomethacin and the internal standard was verified in the following procedures. Methanol containing indomethacin and internal standard was injected into the chromatograph in order to obtain the retention times each day. Furthermore, during development of the assay, plasma samples used for calibration curves were diluted with an equal volume of methanol containing the same concentration of indomethacin and internal standard at each calibration point.

The column was cleaned with acetonitrile at the end of each day and monthly with tetrahydrofuran. The column was stored in methanol.

Results

The indomethacin assay was linear for 0.5 ml of plasma using area ratios for 50 µg/l to 10 mg/l, but a four point calibration from 0.15 mg/l to 1.0 mg/l was used (fig. 1), since the patient levels of indomethacin usually fell within this range. The assay was repeated 10 times with a linear calibration, similar to that in figure 1, obtained for the indomethacin level in 0.5 ml of plasma. The inter-assay coefficients of variation at each point in the 10 calibration curves were: 0.15 mg/l, 8.49%; 0.30 mg/l, 6.48%; 0.50 mg/l, 5.10%; and 1.00 mg/l, 2.22%. The intra-assay coefficients of variation at the 4 calibration points for 2 injections each of duplicate points (assay variation) and for 10 injections of the same standard (integration variation) are shown in table 1. The recovery of both indomethacin and standard was quantitative (tab. 2). Results of repeat analysis following storage of samples at −70 °C for 2 weeks did not vary from those obtained originally. These results were all obtained using 0.5 ml of plasma and a final elution of 1.5 ml of methanol from the C_{18} Sep-Pak. The assay was also linear for indomethacin in concentrations of 0.1 mg/l to 10 mg/l in 0.5 ml of human plasma using peak heights from a strip chart recorder (data not shown).

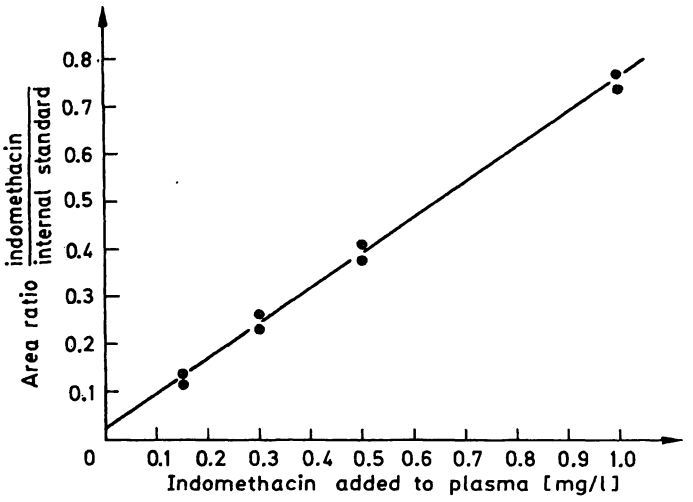

Fig. 1. Area ratio of the indomethacin to the internal standard versus the concentration of added indomethacin. Each calibration point was processed in duplicate (dark circles) as outlined in the Experimental Section and plotted as shown.

Tab. 1. Intra-assay coefficients of variation in the indomethacin HPLC assay[1])

	Coefficients of variation (%)	
Indomethacin (mg/l)	Duplicate values, duplicate points[2])	Ten values, same point[3])
0.15	7.51	8.15
0.30	6.32	6.29
0.50	4.41	5.47
1.00	2.05	5.39

[1]) See Experimental Section for experimental conditions.
[2]) The mean ± SD of duplicates determined in duplicate tubes (n = 4) for each concentration was used to calculate the coefficient of variation.
[3]) The mean ± SD of ten analyses (n = 10) of the same tube for each concentration was used to calculate the coefficient of variation.

Tab. 2. Experimental recovery of indomethacin and internal standard[1])

Indomethacin (mg/l)	Indomethacin Recovery[2]) (%)	Standard Recovery[2]) (%)
0.15	102	99
0.30	100	96
0.50	109	103
1.00	102	98

[1]) The same amount of internal standard (1 µg) and varying amounts of indomethacin were added to both 1.5 ml of methanol and 0.5 ml of plasma. The plasma was processed as outlined in the Experimental Section. Elution of internal standard and indomethacin from Sep-Pak was carried out with 1.5 ml of methanol. The area counts of internal standard and indomethacin recovered in the processed samples was compared directly with the respective area counts in the unprocessed methanol.
[2]) Mean of duplicates.

The Sep-Pak removed contaminants eluting in the flushthrough and also just before the internal standard. Despite column cleaning, without Sep-Pak processing the internal standard would start to elute on the tail of contaminants within 30–50 analyses, making accurate quantitation increasingly difficult. The Sep-Pak cartridges improved sensitivity by stabilizing the baseline detected by the monitor and integrator. Processing 8 calibration curve samples and 14 patient plasma samples followed by HPLC analyses required a total of 16 hours.

Preliminary results have indicated that the assay sensitivity (smaller concentrations of indomethacin in plasma) can be increased by concentrating the final 1.5 ml of methanol 3–5 fold from the Sep-Pak cartridge (fig. 2). We have also shown in preliminary experiments that it is possible to utilize 0.1 ml of plasma, provided the final 1.5 ml of methanol are concentrated 3–5 fold. Processing five different samples of plasma which did not contain either indomethacin or internal standard indicated that there were no interfering substances present in the chromatogram for these two materials. Processing plasma which contained either the internal standard or the indomethacin indicated that these materials were recovered quantitatively from the Sep-Pak cartridge. Analysis of methanol containing indomethacin and internal standard yielded the same chromatogram before (fig. 3) and after passage over the Sep-Pak cartridge. Analysis of those plasma samples obtained from patients prior to administration of indomethacin (fig. 4) yielded no indomethacin (n = 10) compared to samples obtained following indomethacin therapy. Haemolyzed plasma can be used since no interfering substances were detected after processing.

The identification of indomethacin and the internal standard was verified by both coinjection and dilution of the calibration curve samples with an equal volume of methanol containing the same concentration of indomethacin and internal standard at each calibration point. Analysis by HPLC indicated that the rest of the peaks in the chromatogram were diluted to approximately one-half of their initial area counts, while those of internal standard and indomethacin remained the same indicating identity.

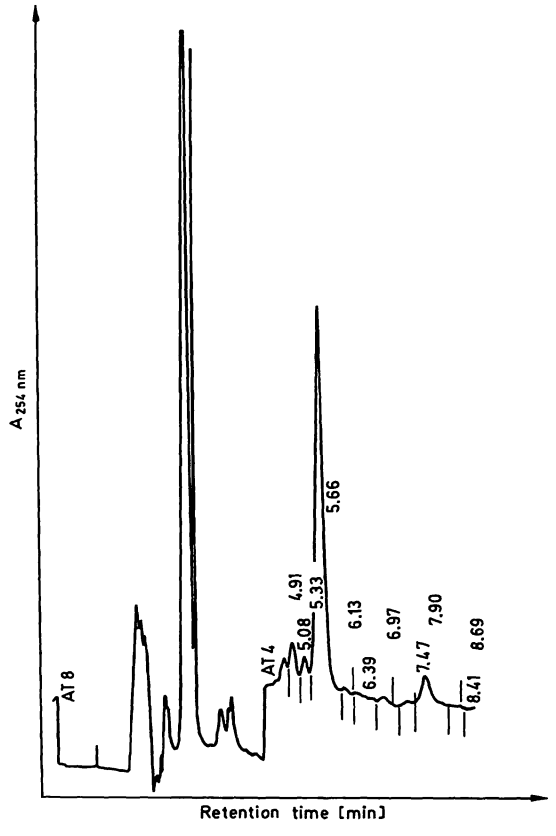

Fig. 2. Chromatogram of 0.5 ml of plasma (48 hours after 3 doses of indomethacin at 12 hour intervals) processed with Sep-Pak as outlined in the Experimental Section. The final 1.5 ml of methanol containing the internal standard and indomethacin was concentrated about 2 fold. The absorbance (0.020 absorbance units full scale) at 254 nm was recorded by the integrator on the ordinate, while peak retention times are recorded in the abscissa. The internal standard eluted at 5.66 minutes with 40 597 area counts, while indomethacin eluted at 7.90 minutes with 5289 area counts (area ratio 0.130, 0.180 mg/l indomethacin).

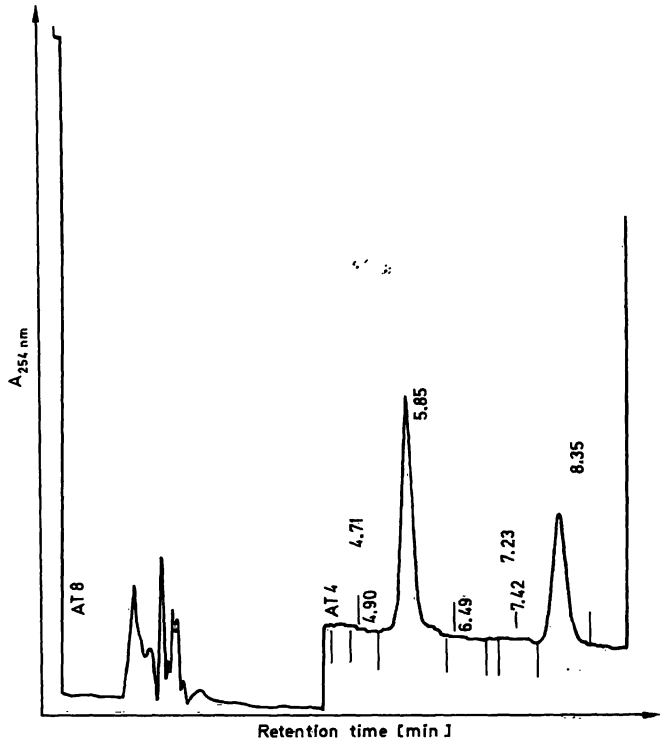

Fig. 3. Chromatogram of 1.0 µg of internal standard and 0.5 µg of indomethacin added to 1.5 ml of methanol (corresponding to a plasma calibration point of 1.0 mg/l indomethacin). The sample was subjected to analysis by HPLC as outlined in the Experimental Section without processing over Sep-Pak. The absorbance (0.020 absorbance units full scale) at 254 nm was recorded by the integrator on the ordinate, while peak retention times were recorded on the abscissa. The internal standard eluted at 5.85 minutes with 23 926 area counts, while indomethacin eluted at 8.35 minutes with 16 528 area counts.

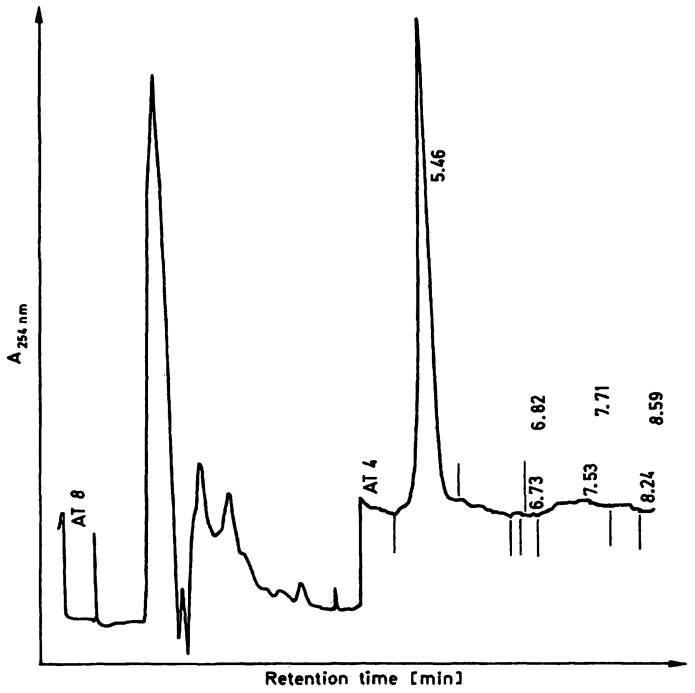

Fig. 4. Chromatogram of 0.5 ml of indomethacin free plasma treated with 2.0 µg of internal standard (twice the usual amount). The sample was processed with Sep-Pak as outlined in the Experimental Section. The absorbance (0.020 absorbance units full scale) at 254 nm was recorded by the integrator on the ordinate, while peak retention times were recorded on the abscissa. The internal standard eluted at 5.46 minutes with 38721 area counts. Indomethacin eluted at 7.67 minutes in calibration curve samples. There were 1097 area counts associated with the peak at 7.71.

Discussion

Modification of an HPLC assay for indomethacin using Sep-Pak has resulted in increased sensitivity, lengthened column life and improved reproducibility with less frequent column cleaning. Though the use of Sep-Pak requires additional time, some samples can be processed while others are being analyzed by HPLC. Thus, the overall time involved is not much longer than that required in other reported assays which do not utilize Sep-Pak.

To date, we have used this method to analyze 112 samples from 16 patients with patent ductus arteriosus using 10 calibration curves with 4 points each performed in duplicate. Patients were treated with indomethacin several times during the course of 72 hours. A peak plasma indomethacin level of 0.500 mg/l usually resulted in closure of the duct (unpublished observations). Reproducibility has been quite satisfactory and the initial column is still in use. Furthermore, since many impurities are removed by Sep-Pak, the sensitivity of the monitor could be higher. This would result in even greater sensitivity since larger area counts would be obtained.

The methanol employed for elution of the Sep-Pak bound indomethacin and internal standard can be concentrated should greater sensitivity be necessary. Alternatively, indomethacin in as little as 100·µl of plasma can be assayed with proper adjustment of the internal standard and initial reagents followed by a 3—5 fold final methanol effluent concentration. Less methanol could probably be used to elute from the Sep-Pak followed by less concentration; we are currently investigating this approach. Decreasing the plasma volume requirement for analysis is of great significance for premature infants. The 1 ml of blood required to generate 300—500 µl of plasma is equivalent to 2.5% of the circulating blood volume of a 500 g premature infant.

Several other methodological observations appear to have improved our results. Various concentrations and combinations of acetic acid, acetonitrile, and methanol were used for processing the plasma samples both initially and with Sep-Pak in order to develop the assay. Acidification of the processed plasma and Sep-Pak cartridge with acetic acid was necessary in order to quantitatively bind the indomethacin and the internal standard to Sep-Pak. In each new lot of Sep-Pak cartridges utilized, verification was made that all of the indomethacin and internal standard were recovered in 1.5 ml of methanol. Occasionally, a minor adjustment may be necessary (± 0.2 ml), depending on the lot of Sep-Pak. The use of such solvents as ethyl ether or tetrahydrofuran during the extraction or elution procedures was avoided since concentration of these solvents can lead to production and accumulation of explosive peroxides. Emulsion producing solvents were not utilized. Finally, the internal standard, 1-(*p*-flurobenzoyl)-5-methoxy-2-methylindole acetic acid (20) has a fluorine substituted for the chlorine present in indomethacin, providing the internal standard with properties (molecular weight, ionic charge and solubility) similar but not indentical to indomethacin. This similarity may have helped in obtaining quantitative recoveries of both indomethacin and the internal standard from Sep-Pak.

Conclusion

In summary, an HPLC assay for indomethacin in small volumes of plasma has been described utilizing Sep-Pak for processing samples. The recovery of indomethacin and the internal standard was quantitative. As a result, the assay is ideal for both clinical management as well as investigational study of plasma prostaglandins and their inhibition in premature infants. Further, preservation of the column using Sep-Pak provides a cost benefit which should, in practical terms, permit more frequent use of this analytical technique.

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